Protein Science (1994), *3*:549–556. Cambridge University Press. Printed in the USA.

The importance of anchorage in determining a strained protein loop conformation

ALEC HODEL,[1,2] ROGER A. KAUTZ,[1,3] DAVID M. ADELMAN,[1] AND ROBERT O. FOX[1,2]

[1] Department of Molecular Biophysics and Biochemistry and [2] The Howard Hughes Medical Institute, Yale University, New Haven, Connecticut 06511

(RECEIVED November 18, 1993; ACCEPTED February 4, 1994)

Abstract

We examine the role of the conformational restriction imposed by constrained ends of a protein loop on the determination of a strained loop conformation. The Lys 116–Pro 117 peptide bond of staphylococcal nuclease A exists in equilibrium between the *cis* and *trans* isomers. The folded protein favors the strained *cis* isomer with an occupancy of 90%. This peptide bond is contained in a solvent-exposed, flexible loop of residues 112–117 whose ends are anchored by Val 111 and Asn 118. Asn 118 is constrained by 2 side-chain hydrogen bonds. We investigate the importance of this constraint by replacing Asn 118 with aspartate, alanine, and glycine. We found that removing 1 or more of the hydrogen bonds observed in Asn 118 stabilizes the *trans* configuration over the *cis* configuration. By protonating the Asp 118 side chain of N118D through decreased pH, the hydrogen bonding character of Asp 118 approached that of Asn 118 in nuclease A, and the *cis* configuration was stabilized relative to the *trans* configuration. These data suggest that the rigid anchoring of the loop end is important in establishing the strained *cis* conformation. The segment of residues 112–117 in nuclease A provides a promising model system for study of the basic principles that determine polypeptide conformations. Such studies could be useful in the rational design or redesign of protein molecules.

Keywords: NMR; protein conformation; proline isomerism; site-directed mutagenesis; staphylococcal nuclease

Protein molecules generally adopt a tertiary structure in which backbone and side-chain conformations are arranged in local energy minima. Strained conformations such as *cis* peptide bonds or eclipsed side-chain rotamers are rarely found. However, in several well-refined protein structures, examples of such locally strained geometries have been observed, usually involving a residue in the enzyme active site (Herzberg & Moult, 1991). Clearly in such cases, some kind of stress or deforming force inherent to the structure must cause the resulting strain or deformation. Although the quality of the crystal structures involved leaves little doubt concerning the correctness of these observations, mechanisms by which the stress is imposed in order to favor locally strained conformations remain unclear.

Several examples exist in short peptide molecules where conformational strain, such as a *cis* peptide bond, is induced through conformational restriction, such as covalent linkage. For example, the cyclic hexapeptide analog of somatostatin, c[Pro-Phe-D-Trp-Lys-Thr-Phe], contains a Phe–Pro *cis* peptide bond "bridging" the ends of the peptide (Kessler et al., 1983; Huang et al., 1992). The stress is induced by the combination of the restriction of the peptide conformation space by rigid anchorage of the ends of the chain, and the restriction of conformation space imposed by its amino acid sequence. By extension, a strained conformation in a protein molecule would also be a product of conformational restraints and limited intrinsic conformation space. However, many times the anchorage of a peptide segment in a protein is not constrained by a single covalent linkage but consists of a number of cooperative low energy interactions, such as hydrogen bonds and Van der Waals interactions, specifically arranged by the protein fold.

We have examined a strained polypeptide segment in strain Foggi staphylococcal nuclease A, the Ca^{2+}-dependent nuclease of *Staphylococcus aureus*. Staphylococcal nuclease, a small protein (149 residues) lacking disulfide bridges, has been the subject of many early protein folding studies (reviewed by Tucker et al., 1979) and of recently renewed interest (Calderon et al., 1985; Serpersu et al., 1985; Shortle & Meeker, 1986; Kuwajima et al., 1991). The crystal structures of nuclease (Hynes & Fox, 1991) and the nuclease-Ca^{2+}-pdTp complex (Cotton et al., 1979; Loll & Lattman, 1989) have been refined to high resolution. NMR studies of nuclease have revealed a slow exchange

Reprint requests to: Robert O. Fox, Department of Molecular Biophysics and Biochemistry, 266 Whitney Avenue, Bass Center, Room 432, P.O. Box 208114, Yale University, New Haven, Connecticut 06520-8114; e-mail: fox@yalevms.bitnet.

[3] Present address: Department of Chemistry, Room 18-020, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139.

between 2 folded conformations observed as multiple resonances for each of the His 8, His 121, and His 124 $H^{\epsilon 1}$ protons (Fox et al., 1986). This conformational heterogeneity was shown to be due to a mixture of *cis* and *trans* isomers at the Lys 116–Pro 117 peptide bond in an equilibrium that favors the *cis* configuration with a fractional occupancy of 90% (Evans et al., 1987, 1989; Wang et al., 1990). Unfolded nuclease and a peptide analog of this segment favor the *trans* isomer of the Lys 116–Pro 117 peptide bond (Evans et al., 1987; Raleigh et al., 1992). Thus, the *cis* configuration of the loop found in the native molecule is a strained element.

We have previously proposed that the stress which causes the strained *cis* 116–117 peptide bond originates in the anchorage of the ends of the loop segment of residues 112–117 by residues Val 111 and Asn 118 (Hodel et al., 1993). Evidence for the identification of Val 111 and Asn 118 as the anchor points is found in numerous X-ray crystal structures of nuclease variants with substitutions in the loop region. Figure 1A and Kinemage 1 show the crystal structures of nuclease A (Hynes & Fox, 1991) and its variants, K116A, K116G (Hodel et al., 1993), K116E, K116D (Hodel et al., in prep.), P117G, P117A, and P117T (Hynes et al., 1994). These 8 structures represent 5 different loop conformations, including both *cis* and *trans* 116–117 peptide bonds, each of which have identical protein structures outside of residues 112–117 (excluding a disordered loop of residues 44–51). In each structure, residues Val 111 and Asn 118 adopt identical conformations in which these 2 residues participate in the same interactions with the rest of the protein context. Val 111 is buried within the protein surface making multiple intramolecular packing contacts, as well as forming several backbone hydrogen bonds to the nearby β-sheet (Kinemage 2). Asn 118 is rigidly fixed by hydrogen bonds between its side chain and nearby residues of the protein structure. The amide nitrogen proton forms a hydrogen bond to the backbone carbonyl oxygen of residue

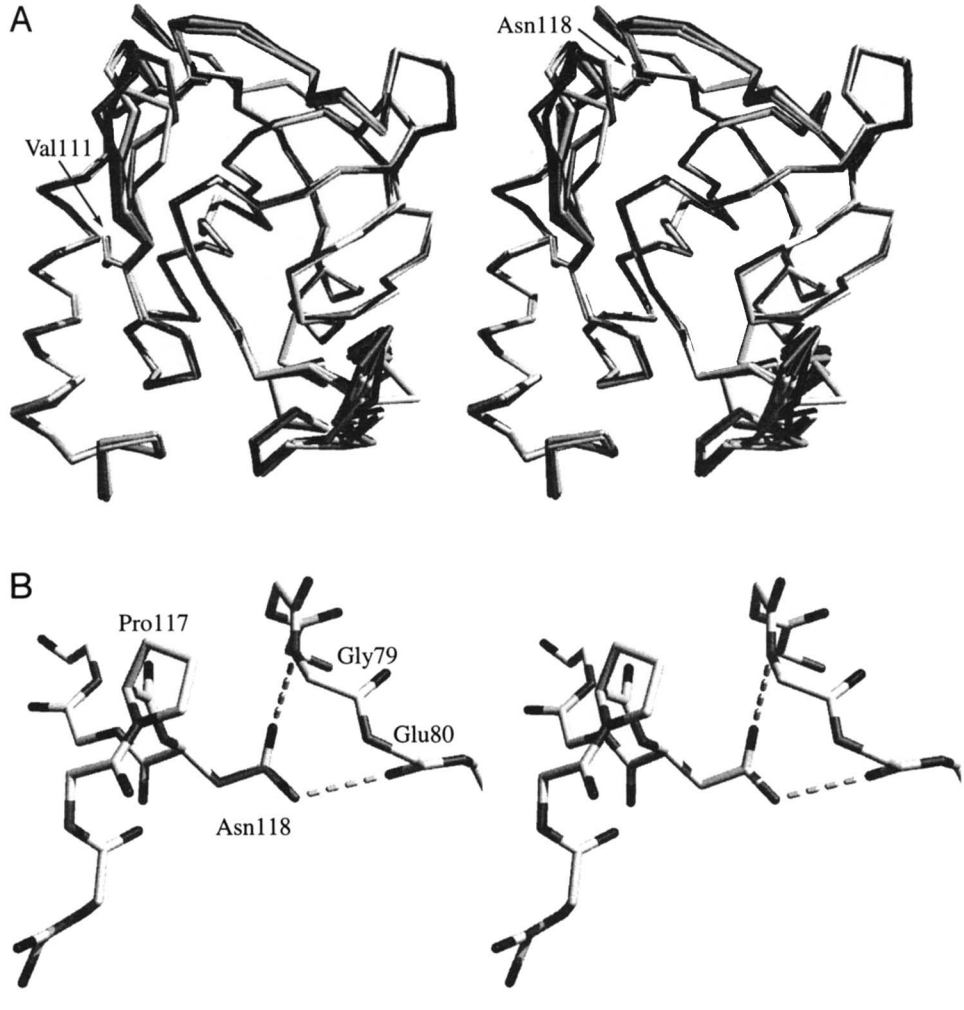

Fig. 1. Anchorage of the residue 112–117 loop in nuclease A and its variants. **A:** Backbone trace for the superimposed X-ray crystal structures of nuclease A and 7 variants, K116A, K116G, K116E, K116D, P117G, P117A, and P117T. These 8 proteins represent 5 different conformations in the 112–117 loop. The local conformational changes that occur in each variant are confined to the 112–117 loop by the anchoring residues, Val 111 and Asn 118. These residues are shown with their side chains. Each of the variant structures were superimposed over the nuclease A structure by minimizing the differences between the coordinates of the α-carbons of all residues except those in the loop of mutation (residues 111–119) and those of a disordered loop (residues 44–51). **B:** Details of the nuclease A structure near Asn 118. The backbone atoms of residues 77–80 and 114–120 are shown along with the side-chain atoms of Pro 117 and Asn 118. Carbon atoms are rendered in white, nitrogen atoms are light gray, and oxygen atoms are dark gray. The 2 anchoring hydrogen bonds of Asn 118, from the δ-oxygen to the backbone nitrogen of Gly 79 and from the δ-nitrogen to the backbone carbonyl oxygen of Glu 80, are shown in dashed lines.

80, and the δ-oxygen of Asn 118 bonds to the backbone nitrogen of residue 79 (see Fig. 1B and Kinemage 3). The conservation of these interactions through all of these mutations suggests that they form the basis for anchorage of the loop ends.

Aside from these endpoints, there is little specific interaction between the loop of residues 112–117 and the rest of the protein context. The only other contacts made between this loop and the rest of the protein occur between Pro 117 and Gly 79 from a nearby loop, and Val 114, whose side chain is oriented toward the protein and packs loosely with the side chains of Leu 38 and Glu 122. The side chains of Tyr 113, Tyr 115, and Lys 116 all extend into the solvent and have no contact with the rest of the protein.

We suggest that the anchoring residues, Val 111 and Asn 118, force the ends of the loop segment to positions that allow only strained backbone conformations, the most favorable of which is the native conformation with a *cis* 116–117 peptide bond. There are several lines of evidence supporting this hypothesis. Mutations in the loop segment that reduce the conformational restrictions of this segment, such as K116G (Hodel et al., 1993) and P117G (Evans et al., 1987; Hynes et al., 1994), increase the fraction of the *trans* population while also increasing the stability of the protein. Mutations within the loop that do not change the intrinsic backbone conformation space of the protein, such as K116A (Hodel et al., 1993), have little effect on the stability or the *cis/trans* equilibrium. If the protein is destabilized through mutation outside of the loop (Alexandrescu et al., 1990) or through solvent conditions (Alexandrescu et al., 1989) the fraction of the *trans* configuration increases. It is our hypothesis that destabilizing the protein reduces the rigidity of the loop end anchorage, allowing *trans* conformations with less strain to appear.

Here we wish to test the importance of the anchorage of the loop ends in the *cis/trans* equilibrium through site-directed mutagenesis. Asn 118 of nuclease was selected for mutagenesis because the only anchoring interactions in which it participates involve the side-chain amide group. The Asn 118 side chain both donates a hydrogen bond, from the δ-nitrogen to the backbone carbonyl oxygen of Glu 80, and accepts a hydrogen bond, from the amide nitrogen of Gly 79 to the δ-oxygen (see Fig. 1B and Kinemage 3). We replaced Asn 118 with aspartate, alanine, and glycine. Aspartate is structurally very similar to asparagine, but at neutral pH it can only form one of the anchoring hydrogen bonds seen in Asn 118, namely the accepted bond from Gly 79 to the δ-oxygen. However, at lower pH the protonated carboxyl group assumes hydrogen bonding characteristics that are very similar to asparagine. In the protonated state, the aspartate side chain could form both anchoring hydrogen bonds and adopt a *cis/trans* equilibrium similar to that of nuclease A. Alanine cannot form either of the side-chain hydrogen bonds, but it has similar limits on its backbone conformation to those of asparagine. Glycine cannot form side-chain hydrogen bonds, and it has considerably more backbone conformational freedom than asparagine.

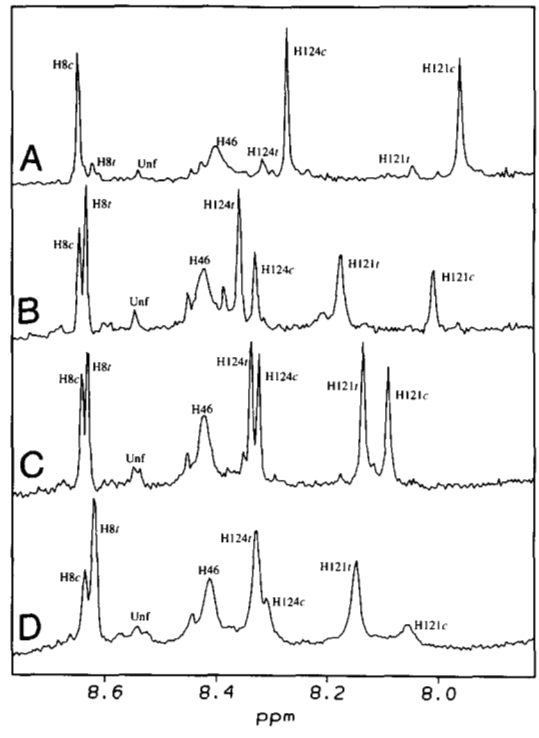

Fig. 2. Low field region of 490-MHz ^{1}H NMR spectra showing resonances of the 4 histidine $H^{\epsilon 1}$ of (**A**) staphylococcal nuclease A, (**B**) the nuclease variant N118D, (**C**) N118A, and (**D**) N118G. The backbone amide protons have been exchanged for deuterons, revealing the histidine $H^{\epsilon 1}$ resonances. These resonances are labeled by residue number suffixed with *c*, denoting the *cis* resonance, and *t*, denoting the *trans* resonance. The peaks corresponding to histidine residues in unfolded protein are labeled Unf. Samples are in 200 mM acetic-d_3-acid-d-buffered D_2O, pH* 5.3. All spectra were taken at 40 °C on the Yale-490MHz in the Chemistry Instrumentation Center at Yale University.

Results

Equilibrium constants and melting temperatures

The histidine $H^{\epsilon 1}$ proton region of the NMR spectrum of each variant is shown in Figure 2. As with nuclease A, 2 resonances appear for the protons of His 8, His 121, and His 124. In nuclease A, each of these resonances corresponds to a different isomerization state of the Lys 116–Pro 117 peptide bond (Evans et al., 1987, 1989). The assignments of the variant histidine $H^{\epsilon 1}$ resonances were inferred from the spectrum of the wild-type protein (Alexandrescu et al., 1988; Kautz et al., 1990), assuming that the order of the histidine chemical shifts is conserved. These tentative assignments were confirmed through titration with the nucleotide inhibitor, pdTp, and through magnetization transfer. The addition of Ca^{2+} and pdTp to a solution of wild-type nuclease (Evans et al., 1989) and the variants K116A and K116G (Hodel et al., 1993) stabilized the *cis* conformation in all 3 proteins. Assuming that this effect is conserved in the Asn 118 variants, solutions of each protein were titrated with Ca^{2+} and the inhibitor. As shown in Figure 3, the proposed *cis* resonances of the N118D variant increased while the *trans* resonances decreased, confirming their assignments. Similar results were obtained for the N118A and N118G variants (data not shown). Magnetization transfer experiments (Evans et al., 1989) were performed on selected histidine protons to confirm conformational exchange (data not shown).

Curve fitting of the histidine region of the NMR spectra (see Fig. 2) yielded the equilibrium constant and the free energy change for the *cis/trans* equilibrium of each protein (see Table 1). The *trans* configuration is more stable in each of the 3

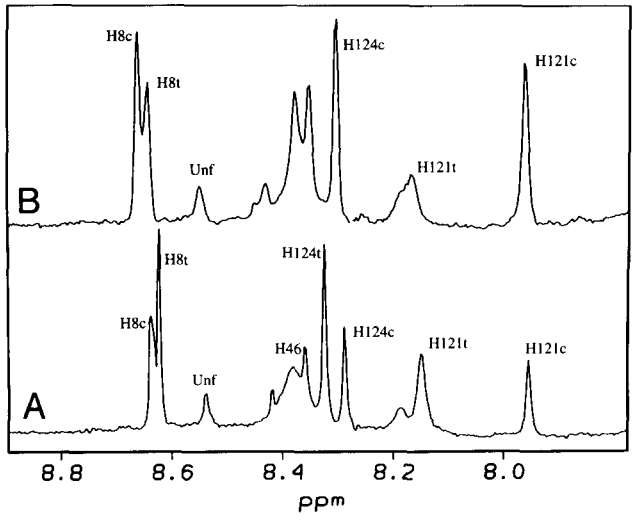

Fig. 3. Effect of ligand binding on the ^{1}H NMR spectrum of variant N118D. Both spectra taken at pH* 5.3, 40 °C, in 200 mM deuterated acetate-buffered D_2O. **A:** No added ligands. **B:** 10 mM $CaCl_2$ and 3 mM 3′,5′-diphosphothymidine (pdTp) added. Intensity can be seen to shift from the *trans* form to the *cis* form resonances as inhibitor is added.

variants where the amino acid at position 118 was altered. Both N118D and N118G populate the *trans* conformation at 63% occupancy with a $\Delta\Delta G_{cis\rightarrow trans}$ of −1.36 kcal/mol relative to nuclease A. The fraction of *trans* molecules in N118A is only 59% with a $\Delta\Delta G_{cis\rightarrow trans}$ of −1.2 kcal/mol from nuclease A.

The melting temperatures of each protein are also shown in Table 1. T_m was defined as the temperature where the unfolded His $H^{\epsilon 1}$ resonances contained half of the integrated histidine $H^{\epsilon 1}$ proton area (see Materials and methods). Each of these variants is less stable against thermal denaturation than the wild-type protein.

pH series

The histidine region of the NMR spectrum of nuclease A and N118D are shown for various pH values in Figure 4. The histidine chemical shifts are sensitive to pH and shift downfield as the pH decreases. The magnitude of this effect is different for each histidine and even for the same histidine in different protein conformations. In Figure 4, the *cis* His 121 resonance of nuclease A shifts downfield more rapidly with decreasing pH than the *trans* resonance resulting in the crossing of the resonances at pH 4.4. As the protein is destabilized by the lowering pH, the resonances for the unfolded state increase in intensity. At pH 3.8, the proteins are mostly unfolded, and the folded histidine resonances were too small to resolve and fit accurately.

The relative areas of the *cis* and *trans* resonances of His 121 were used to calculate the equilibrium constant at each pH value. The results of this calculation are shown in Figure 5. The data show that the *trans* configuration of nuclease A is stabilized relative to the *cis* configuration as the pH is lowered. In N118D, the opposite effect is seen. The *cis* configuration appears to be stabilized relative to the *trans* configuration as the pH decreases. For each protein, the shifts in the equilibrium are significant compared to the errors in the measurement.

Table 1. *Thermodynamic measurements*[a]

Protein	% *Cis* (at 30°C)	$\Delta G_{cis\rightarrow trans}$ (kcal/mol)	T_m (°C)
Nuclease A	87.7 (0.4)	1.2 (0.02)	49 (1)
N118D	31.8 (0.4)	−0.45 (0.03)	46 (1)
N118A	44.8 (0.4)	−0.12 (0.03)	43 (1)
N118G	27.7 (0.4)	−0.56 (0.03)	44 (1)

[a] Errors for these values are given in parentheses.

Discussion

Variants are less stable and adopt a greater fraction of trans conformations

Each of the nuclease variants—N118G, N118A, and N118D—is less stable than the wild-type protein, and each of these variants favors the *trans* 116–117 peptide bond over the wild-type *cis* isomer. This suggests that the 2 hydrogen bonds of the native Asn 118 side chain are important to the overall stability of the protein and to the stability of the *cis* configuration over that of the *trans* configuration. Each variant is examined in detail below.

N118G

N118G cannot make the 2 hydrogen bonds found between Asn 118 and the residue 79–80 loop (see Fig. 1B and Kinemage 3). The loss of this constraint at the C-terminal end of the 112–117 loop should allow for much greater flexibility of the loop. This flexibility is further increased by the lack of steric restriction in the conformation of Gly 118. We hypothesize that this increase in flexibility at the C-terminal end reduces the stress imposed on the 112–117 loop by the rest of the protein. Without the anchorage at residue 118, the loop is allowed to find a *trans* conformation that is less strained than the configuration of the wild-type protein.

The 112–117 loop still exhibits some strain in N118G, as the equilibrium (27% *cis*) favors the *cis* conformation more than the unfolded nuclease (Evans et al., 1987), a model peptide of the loop segment (Raleigh et al., 1993), or the mutant K116G (Hodel et al., 1993) (all 20% *cis*). Thus the residues further down the chain must restrict the position of the C-terminal end of the loop such that stress is still imposed upon the 112–117 segment.

N118A

Similar to N118G, N118A cannot make the anchoring hydrogen bonds of the wild-type asparagine; however, the alanine at residue 118 is sterically restricted, relative to glycine, by its β-methyl group. This restriction reduces the number of conformations with a *trans* Lys 116–Pro 117 peptide bond, which the 112–117 loop may sample, possibly forbidding favorable conformations allowed in N118G. This possibility is consistent with the decrease in the stability of N118A compared to N118G. The replacement

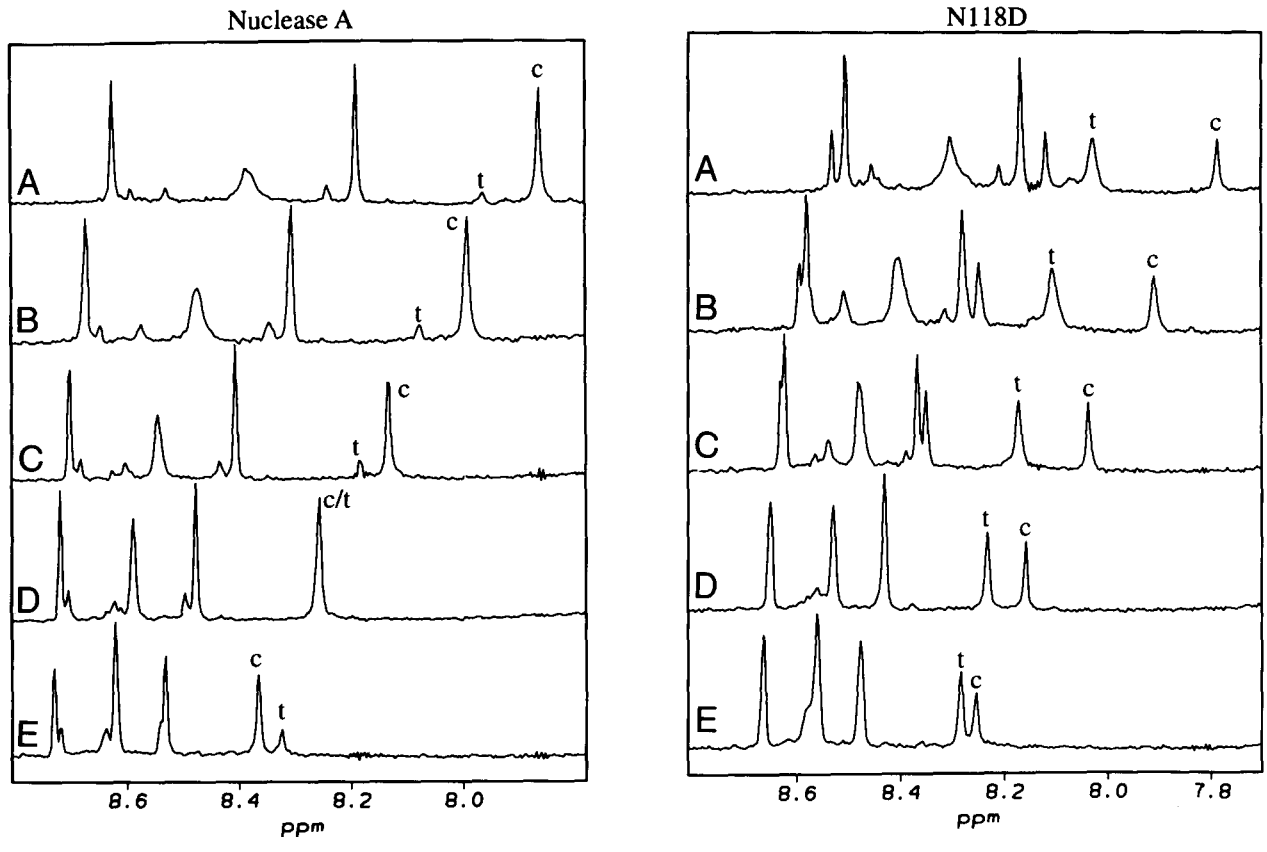

Fig. 4. Effect of changing pH on the ^{1}H NMR spectrum of nuclease A and variant N118D. All spectra taken at 30 °C in 20 mM deuterated acetate, 20 mM deuterated formate-buffered D_2O. **A:** pH* 5.3. **B:** pH* 5.0. **C:** pH* 4.7. **D:** pH* 4.4. **E:** pH* 4.1. In each spectrum, the resonances for His 121 are labeled c, for the *cis* resonance, and t, for the *trans* resonance.

of a glycine residue with an alanine residue should decrease the configurational entropy of the protein in both the folded and unfolded state. If this entropic change affects the folded and unfolded state equally, there would be no difference in the entropic contribution to $\Delta G_{\text{denaturation}}$ for N118G and N118A. However, one would expect that this mutation would have a more pronounced entropic effect on the unfolded state due to the conformational restrictions inherent to the native state. If the entropic effect of the mutation is greater in the unfolded state, $\Delta G_{\text{denaturation}}$ of N118A would be greater than that of N118G. Because N118A is less stable to denaturation than N118G, the alanine at position 118 must adopt a configuration that is enthalpically unfavorable compared to that of the glycine. The greater steric hindrance of the alanine could then be responsible for the increased relative stability of the *cis* configuration in N118A compared to the equilibrium in N118G.

N118D

N118D could theoretically satisfy 1 of the 2 hydrogen bonds found in the wild-type structure, namely, the bond from the 118 side-chain δ-oxygen to the backbone nitrogen of residue 79. We assume that this hydrogen bond is present in the *cis* conformation, modeling this conformation after that of the wild-type protein. It is unclear whether this hydrogen bond exists in the *trans* form of the protein. $\Delta G_{cis \rightarrow trans}$ is slightly smaller in N118D compared to N118A. One would expect that the family of backbone conformations available to both alanine and aspartate would be similar. Thus, the primary differences between the conformations of N118A and N118D would originate in the interactions of the aspartate side chain with its environment. Thus, if the single hydrogen bond exists in the *trans* form of N118D, the addition of the hydrogen bond to one of the *trans* conformations available in N118A would have to stabilize this conformation more than the addition of a hydrogen bond to the *cis* conformation, if it is similar in nuclease A, N118A, and N118D. Because the difference in $\Delta G_{cis \rightarrow trans}$ between N118D and N118A is small (0.33 kcal/mol), such a situation is conceivable.

If the Asp 118–Gly 79 hydrogen bond does not exist in the *trans* form of N118D, the difference in $\Delta G_{cis \rightarrow trans}$ between N118A and N118D could be due to the solvation energy of the aspartate side chain. Without this hydrogen bond, the side chain of Asp 118 could easily extend into the solvent. The δ nitrogen and oxygen of Asn 118 are nearly buried in the conformation found in wild-type nuclease A. If we assume that the *cis* conformation of N118D is similar to the wild-type conformation, then the solvated *trans* conformation of N118D could be stabilized in favor of the *cis* conformation, which would bury the charged δ oxygens.

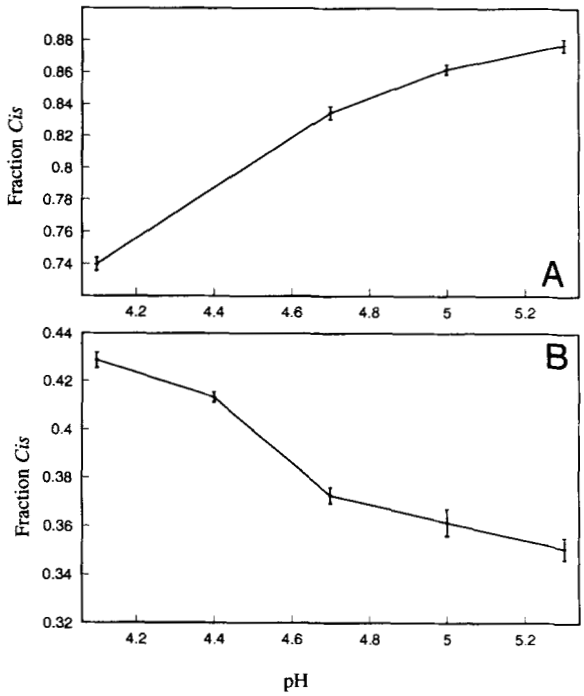

Fig. 5. Graphs of the fraction of the *cis* form found in **(A)** nuclease A and **(B)** N118D, along with the errors in this measurement, as a function of pH. The fraction of the *cis* form was calculated from the areas of the well-resolved His 121 resonances of the spectra shown in Figure 4. Values and errors were calculated by nonlinear least-squares curve fitting (see Materials and methods). No value is reported for nuclease A at pH 4.4 because the His 121 resonances were not resolved.

Protonating D118 stabilizes the cis conformation

Protonation of the D118 side chain gives the aspartate hydrogen bonding character very similar to that of asparagine. A protonated aspartate at position 118 could accommodate both the hydrogen bond to the amide nitrogen of Gly 79 through the unprotonated oxygen, and the hydrogen bond to the oxygen of Glu 80 through the protonated oxygen. Thus, one would expect that a decrease in the pH toward the pK_a of the aspartate side chain would cause the $\Delta G_{cis \rightarrow trans}$ of N118D variant to approach that of the wild-type protein.

We tested this hypothesis by measuring the *cis/trans* equilibrium in both the wild-type protein and the variant N118D as a function of pH between 4.1 and 5.3. In the wild-type protein, the *trans* configuration is stabilized relative to the *cis* configuration as the pH is decreased. This effect was also seen in earlier work by Alexandrescu et al. (1989) and can be explained by our stress and strain model (Hodel et al., 1993). Establishing the *cis* conformation depends on the rigid anchoring of the end points of the 112–117 loop by the overall fold of the protein. As the protein is destabilized, the ends are not anchored as rigidly, allowing *trans* conformations with less strain to be populated.

In the N118D variant, the *cis* conformation is stabilized relative to the *trans* conformation as the pH decreases. This suggests that the protonated Asp 118 is forming the 2 anchoring hydrogen bonds seen in the wild-type protein, stabilizing the *cis* conformation over the *trans* configuration. Thus, by replacing Asn 118 with aspartate, the rigid anchorage of the C-terminal end of the 112–117 loop is reduced by the removal of at least one of the anchoring hydrogen bonds, resulting in an increase in the fraction of *trans* conformations seen in solution. By protonating the aspartate side chain, the wild-type hydrogen bonding pattern is restored, and the anchor-dependent *cis* conformation is stabilized. If this analysis is correct, N118D is a pH-dependent "conformation switch" where high pH favors the *trans* conformation and low pH favors the *cis* conformation. In reality, the application of this switch is limited by the denaturation of N118D at a pH near the pK_a of an aspartate side chain (~pH 4).

Summary

The nuclease variants N118D, N118A, and N118G test the contribution of the 2 side-chain hydrogen bonds of Asn 118 to the equilibrium between the *cis* and *trans* forms of the residue 112–117 loop. In each case, one or more of the Asn 118 hydrogen bonds were removed and the *trans* configuration was stabilized relative to the *cis* configuration. By protonating the Asp 118 side chain of N118D through decreased pH, the hydrogen bonding character of Asp 118 approached that of Asn 118 in nuclease A, and the *cis* configuration was stabilized relative to the *trans* configuration.

These results, along with other studies, suggest that in the determination of a protein loop segment's conformation, the major contribution of the protein context is the positioning of the loop ends. A search of conformational space for short peptide segments taken from known protein structures has proven successful in modeling the correct polypeptide backbone geometry when the ends of the segment are constrained to join the remaining structure (Chothia et al., 1986; Fine et al., 1986; Moult and James, 1986; Bruccoleri & Karplus, 1987). Wolfson et al. (1993) found that when a loop segment from 1 protein is transferred to a structurally homologous protein, the loop in the chimeric protein retains its original function. Hynes et al. (1989) found that when the β-turn segment from concanavalin A is transplanted into a turn site in staphylococcal nuclease with the same loop end geometry, the turn segment adopts its original conformation.

The segment of residues 112–117 in nuclease A provides a promising model system for study of the basic principles that determine polypeptide conformations. Such studies will be important in the rational design or redesign of protein molecules.

Materials and methods

Preparation of the nuclease variants

The nuclease A gene was subcloned into M13mp18 to produce single-stranded template DNA. The N118G mutant was prepared by primer-directed mutagenesis using synthetic oligonucleotides (Zoller & Smith, 1983). Plaques were screened by differential hybridization using 5′-^{32}P-labeled oligonucleotides and sequenced to verify the mutation. The N118A and N118D mutants were prepared by PCR mutagenesis using synthetic oligonucleotides (Higuchi, 1990) and were sequenced to verify the mutation. The mutant genes were subcloned into the plasmid pAS1 and expressed in *Escherichia coli*, and the protein was prepared as described (Evans et al., 1989).

^{1}H-NMR determination of cis/trans equilibrium

Ten to 30 mg of lyophilized protein was resuspended in D_2O and adjusted to pH* 5.3 (pH* refers to glass electrode meter reading uncorrected for deuterium isotope effects [Bundi & Wüthrich, 1979]). The sample was then heated to 10 °C above the T_m for 5 min to facilitate exchange of labile protons, and any precipitate was removed by centrifugation. The deuterated protein was lyophilized again and resuspended in 0.5 mL of 200 mM acetic-d_3-acid-d buffered D_2O, pH* 5.3, with 1 mM TSP (trimethylsilylpropionate; Aldrich) as a chemical-shift reference. Trace precipitate, if present, was removed by centrifugation before transferring to a 5-mm NMR tube.

The assignments of the $H^{\epsilon 1}$ histidine peaks were performed by comparison to the wild-type protein spectrum. The resonances that corresponded to the *cis* and the *trans* conformations were differentiated by the addition of Ca^{2+} and the competitive inhibitor 3′,5′-diphosphothymidine (pdTp). Conformational exchange between the *cis* and *trans* peaks of individual histidine protons was confirmed through magnetization transfer experiments (Evans et al., 1989). Spectra were transformed and analyzed using the program Felix (Hare Research).

The fraction of molecules with a *cis* Lys 116–Pro 117 peptide bond was obtained from the relative areas of the resolved histidine resonances of spectra acquired at 30 °C. The nonlinear least-squares curve fitting of the Lorentzian lines was performed using the Leverberg–Marquardt algorithm (Press et al., 1986). Errors in the fitting parameters were estimated by applying normally distributed shifts to the experimental data and repeating the curve fitting to yield a distribution of fitting parameters. The ΔG values for the *cis/trans* equilibrium were obtained using $\Delta G = -RT \ln K$, where K is the equilibrium constant for the isomerization from *cis* to *trans*.

Temperature series

NMR spectra of the 3 variants of nuclease in 200 mM acetic-d_3-acid-d-buffered D_2O, pH* 5.3, were prepared as described above. The T_m was first estimated from a series of spectra with 5- or 10- °C temperature increments. The T_m was then accurately determined from a series of at least 10 spectra with 2 °C temperature increments, long temperature equilibration, and adjustment of shims before each acquisition. The relative precision of temperatures in a series was ±0.1 °C; the absolute accuracy of the temperatures of any series is within 0.5 °C.

Areas of histidine resonances of unfolded protein were measured from spectra by curve fitting. The equilibrium constant of folding (K) was determined for each temperature through nonlinear least-squares fitting as described above. These values were plotted with uncertainties on a van't Hoff plot, $\ln(K)$ vs. $1/T$. The T_m and its uncertainty were determined from the $\ln(K) = 0$ intercepts of the best fit and worst acceptable fit lines.

pH series

One hundred eighty milligrams of lyophilized nuclease A and N118D protein was suspended in D_2O and heated to 10 °C above the T_m for 5 min to facilitate exchange of labile protons. The deuterated protein was lyophilized again and resuspended in 3 mL of a buffer composed of 20 mM acetic-d_3-acid-d and 20 mM Na-formate-d. Starting from pH* 5.4, these solutions were titrated with DCl and NaOD to pH* 5.3, 5.0, 4.7, 4.4, 4.1, and 3.8. At each pH* value, a 0.5-mL aliquot was taken for ^{1}H-NMR analysis. The *cis/trans* equilibrium was then determined through curve fitting as above.

Acknowledgments

This work was supported by an NIH grant to R.O.F. (AI23923), by the Howard Hughes Medical Institute, and by shared instrumentation grants to Stanford University and Yale University (NIH RR 03475, NSF DMB8610557, and ACS RD259) for the NMR spectrometers. A.H. is a Howard Hughes Medical Institute Predoctoral Fellow. We thank M.D. Jacobs for helpful discussions.

References

Alexandrescu AT, Hinck AP, Markley JL. 1990. Coupling between local structure and global stability of a protein: Mutants of staphylococcal nuclease. *Biochemistry 29*:4516–4525.

Alexandrescu AT, Mills DA, Ulrich EL, Chinami M, Markley JL. 1988. NMR assignments of the four histidines of staphylococcal nuclease in native and denatured states. *Biochemistry 27*:2158–2165.

Alexandrescu AT, Ulrich EL, Markley JL. 1989. Hydrogen-1 NMR evidence for three interconverting forms of staphylococcal nuclease: Effects of mutations and solution conditions on their distribution. *Biochemistry 28*:204–211.

Bruccoleri RE, Karplus M. 1987. Prediction of the folding of short polypeptide segments by uniform conformational sampling. *Biopolymers 26*:137–168.

Bundi A, Wuthrich K. 1979. ^{1}H-NMR parameters of the common amino acid residues measured in aqueous solutions of the linear tetrapeptides H-gly-gly-X-ala-OH. *Biopolymers 18*:285–297.

Calderon RO, Stolowich NJ, Gerlt JA, Sturtevant JM. 1985. Thermal denaturation of staphylococcal nuclease. *Biochemistry 24*:6044–6049.

Chothia C, Lesk AM, Levitt M, Amit AG, Mariuzza RA, Phillips SEV, Poljak RJ. 1986. The predicted structure of immunoglobulin D1.3 and its comparison with the crystal structure. *Science 233*:755–758.

Cotton FA, Hazen EE Jr, Legg MJ. 1979. Staphylococcal nuclease: Proposed mechanism of action based on structure of enzyme-thymidine 3′,5′-bisphosphate-calcium ion complex at 1.5 Å resolution. *Proc Natl Acad Sci USA 76*:2551–2555.

Evans PA, Dobson CM, Kautz RA, Hatfull G, Fox RO. 1987. Proline isomerism in staphylococcal nuclease characterized by NMR and site-directed mutagenesis. *Nature 329*:2266–2268.

Evans PA, Kautz RA, Fox RO, Dobson CM. 1989. A magnetization-transfer nuclear magnetic resonance study of the folding of staphylococcal nuclease. *Biochemistry 28*:362–370.

Fine RM, Wang H, Shenkin PS, Yarmush DL, Levinthal C. 1986. Predicting antibody hypervariable loop conformations II: Minimization and molecular dynamics studies of MCPC603 from many randomly generated loop conformations. *Proteins Struct Funct Genet 1*:342–362.

Fox RO, Evans PA, Dobson CM. 1986. Multiple conformations of a protein demonstrated by magnetization transfer NMR spectroscopy. *Nature 320*:192–194.

Herzberg O, Moult J. 1991. Analysis of the steric strain in the polypeptide backbone of protein molecules. *Proteins Struct Funct Genet 11*:223–229.

Higuchi R. 1990. Recombinant PCR. In: Innis MA, Gelford DH, Sninsky JJ, White TJ, eds. *PCR protocols: A guide to methods and applications*. San Diego: Academic Press. pp 177–183.

Hodel A, Kautz RA, Jacobs MD, Fox RO. 1993. Stress and strain in staphylococcal nuclease. *Protein Sci 2*:838–850.

Huang Z, He YB, Raynor K, Tallent M, Reisire T, Goodman M. 1992. Main chain and side chain chiral methylated somatostatin analogs: Synthesis and conformational analysis. *J Am Chem Soc 114*:9390–9401.

Hynes TR, Fox RO. 1991. The crystal structure of staphylococcal nuclease refined at 1.7 Å resolution. *Proteins Struct Funct Genet 10*:92–105.

Hynes TR, Hodel A, Fox RO. 1994. Engineering alternative β-turn types in staphylococcal nuclease. *Biochemistry*. Forthcoming.

Hynes TR, Kautz RA, Goodman MA, Gill JF, Fox RO. 1989. Transfer of a β-turn structure to a new protein context. *Nature 339*:73–76.

Kautz RA, Gill JA, Fox RO. 1990. Assignment of histidine resonances in the NMR spectrum of staphylococcal nuclease using site-directed mutagenesis. In: Craik C, ed. *Protein and pharmaceutical engineering*. New York: Wiley-Liss, Inc. pp 1–15.

Kessler H, Bernd M, Kogler H, Zarbock J, Sorensen OW, Bodenhausen G, Ernst RR. 1983. Relayed heteronuclear correlation spectroscopy and conformational analysis of a cyclic hexapeptide containing the active sequence of somatostatin. *J Am Chem Soc 105*:6944–6952.

Kuwajima K, Okayama N, Yamamoto K, Ishihara T, Sugai S. 1991. The Pro 117 to glycine mutation of staphylococcal nuclease simplifies the unfolding-folding kinetics. *FEBS Lett 290*:135–138.

Loll PA, Lattman EE. 1989. The crystal structure of the ternary complex of staphylococcal nuclease, Ca^{2+}, and the inhibitor pdTp, refined at 1.65 Å. *Proteins Struct Funct Genet 5*:183–201.

Moult J, James MNG. 1986. An algorithm for determining the conformation of polypeptide segments in proteins by systematic search. *Proteins Struct Funct Genet 1*:146–163.

Press WH, Flannery BP, Teukolsky SA, Vetterling WT. 1986. *Numerical recipes*. Cambridge, UK: Cambridge University Press.

Raleigh DP, Evans PA, Pitkeathly M, Dobson CM. 1992. A peptide model for proline isomerism in the unfolded state of staphylococcal nuclease. *J Mol Biol 228*:338–342.

Serpersu EH, Shortle D, Mildvan AS. 1985. Kinetic and magnetic resonance studies of effects of genetic substitution of a Ca^{2+} liganding amino acid in staphylococcal nuclease. *Biochemistry 25*:68–77.

Shortle DJ, Meeker AK. 1986. Mutant forms of staphylococcal nuclease with altered patterns of guanidine hydrochloride and urea denaturation. *Proteins Struct Funct Genet 1*:81–89.

Tucker PW, Hazen EE, Cotton FA. 1979. Staphylococcal nuclease reviewed: A prototypic study in contemporary enzymology. IV. Nuclease as a model for protein folding. *Mol Cell Biochem 23*:131–141.

Wang J, Hinck AP, Loh SN, Markley JL. 1990. Two-dimensional NMR studies of staphylococcal nuclease: Evidence for conformational heterogeneity from hydrogen-1, carbon-13, and nitrogen-15 spin system assignments of the aromatic amino acids in the nuclease H124L–thymidine 3′,5′-bisphosphate–Ca^{2+} ternary complex. *Biochemistry 29*:4242–4253.

Wolfson AJ, Kanaoka M, Lau F, Ringe D, Young P, Lee J, Blumenthal J. 1993. Modularity of protein function: Chimeric interleukin 1βs containing specific protease inhibitor loops retain function of both molecules. *Biochemistry 32*:5327–5331.

Zoller ML, Smith M. 1983. Oligonucleotide-directed mutagenesis of DNA fragments cloned into M13 vectors. *Methods Enzymol 100*:468–500.